AFAMRL-TR-81-82

CYTOLOGIC AND BIOCHEMICAL GENETIC EFFECTS OF CHEMICAL CARCINOGENS

RONALD E. RASMUSSEN, Ph.D.

UNIVERSITY OF CALIFORNIA, IRVINE
COMMUNITY AND ENVIRONMENTAL MEDICINE
IRVINE, CALIFORNIA 92717

OCTOBER 1981

AIR FORCE AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

NOTICES

When US Government drawings, specifications, or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data, is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from Air Force Aerospace Medical Research Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with Defense Documentation Center should direct requests for copies of this report to:

Defense Documentation Center
Cameron Station
Alexandria, Virginia 22314

TECHNICAL REVIEW AND APPROVAL

AFAMRL-TR-81-82

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

Michael G MacNaughton

MICHAEL G. MACNAUGHTON, Lt Colonel, USAF, BSC
Deputy Director, Toxic Hazards Division

AIR FORCE/56780/3 November 1981 — 150

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER AFAMRL-TR-81-82	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* CYTOLOGIC AND BIOCHEMICAL GENETIC EFFECTS OF CHEMICAL CARCINOGENS		5. TYPE OF REPORT & PERIOD COVERED Annual Report 7/1/80-6/30/81
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Ronald E. Rasmussen		8. CONTRACT OR GRANT NUMBER(s) F33615-80-C-0512
9. PERFORMING ORGANIZATION NAME AND ADDRESS The Regents of the University of California University of California, Irvine Irvine, CA 92717		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62202F-6302-01-15
11. CONTROLLING OFFICE NAME AND ADDRESS Air Force Aerospace Medical Research Laboratory Aerospace Medical Division, Air Force Systems Command, Wright-Patterson AFB, Ohio 45433		12. REPORT DATE OCTOBER 1981 13. NUMBER OF PAGES 43
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*		15. SECURITY CLASS. *(of this report)* UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*
Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

DNA Repair	Animal Models of Cancer	3-Methylcholanthrene
4-nitroquinoline-1-oxide	Hydrazine	Rats
Dimethylnitrosamine	Nasal Cancer	Hamsters
Chemical Carcinogenesis	Cytogenetics	Mice
Lung Cancer	Benzo(a)pyrene	

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

During the past year, studies have been conducted in three project areas which are:

1. DNA Repair Synthesis as a Possible Indication of Carcinogenesis;
2. Effect of Acute Hydrazine Exposure on Cellular Proliferation, DNA Replication, and DNA Repair in Rodent Lung;
3. Studies in a Rat Lung Tumor Model: Cellular Biochemistry and Cytogenetics

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

The first project is concerned with the relationship between initiation of cancer by chemicals and the damage and repair of DNA in cells and tissues susceptible to the carcinogenic action of the chemicals. The working hypothesis is that interaction of the carcinogen with cellular DNA is a necessary step in carcinogenesis and that this interaction may cause DNA damage which is then followed by DNA repair synthesis. On this basis, one might expect that carcinogen-DNA interactions (and hence DNA repair synthesis) would be most extensive in those tissues and organs susceptible to the carcinogenic effect. Experiments reported here show this to be the case for a known lung carcinogen, 4-nitroquinoline-1-oxide (4NQO). When injected into mice, 4NQO caused DNA damage in the lung which could be detected upon removal of the lungs and incubation in vitro with ^{3}H-thymidine, a tracer for DNA synthesis. Other organs (liver, kidney) showed much lower levels of DNA repair synthesis after 4NQO treatment. Treatment of the mice with dimethylnitrosamine (DMN) induced DNA damage in lung, liver and kidney, which are the targets for this carcinogen. Of these two chemicals, only 4NQO caused DNA damage in spleen lymphocytes in vivo; however, 4NQO did not induce DNA repair synthesis in spleen lymphocytes when they were incubated in vitro with the chemical. DMN did not induce DNA repair synthesis in vivo or in vitro in spleen lymphocytes. The conclusions were that these two chemicals did induce DNA damage and DNA repair synthesis in the target organs for their carcinogenic action; however, further studies are necessary before the extent of this specificity can be determined.

The second project was conducted with the cooperation of the staff of the Toxic Hazards Research Unit at AMRL, WPAFB. Rats and hamsters were exposed by inhalation to hydrazine at 750 ppm for one hour. Groups of animals which had been exposed only once or which had been exposed 10 times were shipped to UC Irvine for study. The factors studied were 1) DNA replication in the lung; 2) DNA repair synthesis in the lung; 3) Cellular replication in the lung and nasal turbinates; and 4) Metabolism of a known lung carcinogen, ^{3}H-benzo(a)pyrene by lung slices from the treated animals. After a single exposure to hydrazine, DNA replication was depressed in the lung of both species, and ^{3}H-benzo(a)pyrene metabolism was depressed in the lung of the rats. By seven days post-exposure, these activities had returned to control levels. There appeared to be no cumulative effect of repeated exposure to hydrazine on these activities. The nasal turbinates of both species sustained substantial damage to the respiratory and olfactory epithelia, with the hamsters appearing to be more affected. By seven days post-exposure, the epithelia in the rats had recovered and appeared nearly normal. However, the olfactory epithelium of the hamsters had not. The results of studies of the animals exposed 10 times to hydrazine are pending.

The third project was initiated during the current year with the goal of developing an animal model in which biochemical and cytogenetic factors could be studied during the process of tumorigenesis. Range-finding studies are underway at the Toxic Hazards Research Unit (THRU) to establish the intratracheal dosage of 3-methylcholanthrene (MCA) necessary to produce a high yield of lung tumors in Fischer 344 rats within 12 months. Using the established dosage, a second group of rats will be treated at the THRU and at intervals, animals will be killed and tissues sent to UC Irvine for analysis. Factors to be studied include ^{3}H-benzo(a)pyrene metabolism by lung and liver enzymes, chromosomal damage in the lung, and cellular proliferation in the lung. Studies during the current year have established the feasibility of shipment of cells and tissues from the THRU to UC Irvine. The major study is expected to begin early in 1982.

PREFACE

This is the first annual report of the Cytology, Cell Biology, and Cytogenetics Section of the Toxic Hazards Research Program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under Contract Number F33615-80-C-0512. This report describes the research activities at UCI from September 1, 1980 through April 30, 1981. During this period, T.T. Crocker, M.D. was Principal Investigator for the contract. R.E. Rasmussen, Ph.D. conducted the studies at UCI. Jean Anderson served as Staff Research Associate and Arthur T. Fong as Research Assistant at UCI. Technical Monitor was Kenneth C. Back, Ph.D., Air Force Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base.

This Section is concerned with the relationship of cytogenetic and biochemical genetic effects of environmental chemicals to their toxic and/or carcinogenic effects in experimental animals and humans. The studies include chromosomal damage, stimulation of cellular proliferation, and molecular damage to cellular DNA as evidence by stimulation of a DNA repair response in tissues of experimental animals exposed in vivo and in vitro to known carcinogenic chemicals.

The goal of the studies is to better understand the mechanisms by which environmental carcinogens cause genetic damage in those organs which are the target tissues for their carcinogenic effect, and to thereby gain additional means of evaluating the potential hazards of exposure of humans to new materials entering the environment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

DNA REPAIR SYNTHESIS AS A POSSIBLE INDICATION OF CARCINOGENESIS

INTRODUCTION

Statement of the Problem

The effects on humans of environmental contaminants cannot, with complete confidence, be deduced from presently available studies of their effects on experimental animals. Therefore, additional methods must be developed to serve as indicators of premutagenic, pre-carcinogenic and pre-teratogenic events that may result from pollutant encounter. Such testing methods must be sensitive, reliable, and should be relatively easy to perform. Further, they must be relevant to the expected hazard, i.e., to the genetic damage that is thought to result in mutation, etc. These requirements have suggested that tests for cytogenetic damage can be developed using peripheral blood cells, and in fact it has been demonstrated that cytogenetic damage can be detected in this way. However, most studies to date have been conducted in the laboratory, and only a very few have been done with animals exposed to environmental hazards.

Nearly all suggested approaches to the testing and evaluation of environmental pollutants in respect to their mutagenic, carcinogenic or teratogenic effects are based (with good reason) on the assumption that the active pollutants exert their effects through direct or indirect modification of the genetic material, DNA. In support of this there are many in vivo and in vitro studies showing that mutagenic and carcinogenic chemicals undergo physical and/or chemical interactions with DNA while structurally similar noncarcinogenic chemicals do not.

A frequently observed consequence of this carcinogen-DNA interaction is that an endogenous, constitutive DNA repair mechanism is called upon to restore the original structure of the DNA molecule. By employing the methods described in detail below, the repair process can be quantified and some information gained about the potency of the carcinogen and/or its mechanism of action.

The fact that a chemical or physical agent elicits a DNA repair response in an exposed cell or tissue is suggestive evidence for its being a mutagen or carcinogen (see below for further discussion). This implies that carcinogens may be detectable by determining whether a test chemical or other material produces a DNA repair response in appropriately exposed cells. Furthermore, it suggests that the presence of a DNA repair response in an animal exposed to a suspect carcinogen may be indicative of possible genetic damage.

In order to establish whether the above speculations can be used to develop practical testing protocols in experimental animals and possibly in humans, a systematic study has been undertaken to correlate in vitro observations of DNA repair synthesis (DRS) in animal cells and tissues with in vivo determination of DRS in the target organ(s) for carcinogenesis and in peripheral lymphocytes of exposed animals. If such correlations are found, protocols may be developed for testing of materials of interest to the USAF/USN for their activity in the induction of DRS in appropriate species.

Specific Aims

This project is concerned with the evaluation of chemical carcinogen-induced DRS in experimental animals as an indication of potentially carcinogenic hits on DNA. Studies are being conducted to determine whether correlations exist between the chemical carcinogen-induced DRS seen in cell and organ cultures in vitro and that produced in the target organ for carcinogenesis and in the peripheral lymphocytes of the exposed animals. If such correlations can be demonstrated, the system may be used to test materials of interest to the USAF/USN for evidence of DRS stimulation in the peripheral lymphocytes of appropriately exposed animals and possibly in suspect target organs for carcinogenesis.

The goal of these studies is to establish whether DRS induction can be used as an aid in determining possible carcinogenic effects in humans of materials of interest to the USAF/USN.

The specific aims of this project are:

1. To elucidate the relationships between chemical carcinogen-induced DRS in target tissues in vivo and in non-target tissues and cells in vivo and in vitro;

2. To determine whether peripheral lymphocytes from experimental animals can serve as indicator cells for carcinogen exposure when the animals have experienced a carcinogen dose known to produce tumors at some incidence.

MATERIALS AND METHODS

Measurement of DNA Replication and Repair Synthesis in Lung and Other Tissues

The method is based on the fact that both DNA replicative synthesis and DNA repair synthesis can, under the appropriate experimental conditions, be quantified by determining the incorporation of ^{3}H-thymidine (^{3}H-dThd) into macromolecular cellular

DNA during an in vivo or in vitro incubation period (Painter and Cleaver, 1969; Cleaver, 1974). In the present experiments, DNA replication and repair synthesis have been measured in tissue slices and in peripheral lymphocytes incubated with ^{3}H-dThd in vitro. The methods are somewhat different, and are described separately.

DNA Replication and Repair Synthesis in Tissue Slices

Lung slices were prepared by first inflating the lungs of treated animals in situ with melted agar (2%, 42-43°C) via a tracheal cannula, and allowing the agar to solidify by placing the excised lung in ice cold saline solution. In order to maintain cellular viability, the agar was dissolved in a balanced salt solution which consisted of Dulbecco's phosphate buffered saline (Grand Island Biological Co., Grand Island, NY), Kaighn's F12K amino acids (Kaighn, 1973), and 1% fetal bovine serum (Grand Island Biological Co.). The same solution, less the agar, was used for chilling the excised lung. Lobes, or pieces thereof, were dissected free of the trachea and heart, and placed in the barrel of a 6 ml plastic syringe from which the luer end had been cut off, and the plunger inserted in the reverse direction. Additional agar was added so that the lung lobes were embedded in a cylinder of agar. The cylinder of agar was then slowly extruded using the plunger, and freehand slices of the lung were cut with a razor blade to a thickness of about 1 mm and accumulated in ice cold saline. Slices of liver and kidney were prepared in a similar manner except that these tissues were not inflated with agar prior to embedding them in agar in the syringe.

The composition of the incubation medium depended on the activity to be measured. DNA replicative synthesis was measured by incubation of the slices in the above salt solution to which was added ^{3}H-dThd at 5 µCi/ml at a specific radioactivity of 50-60 Ci/mmole. For measurement of DNA repair synthesis, the incubation mixtures also contained hydroxyurea (HU) at a concentration of 10^{-2}M. The latter compound strongly inhibits replicative DNA synthesis, but, at the concentration used, has little effect on DNA repair synthesis (Brandt et al., 1972). Tissue slices containing 20-40 mg protein were incubated in a total volume of 2.0 ml of medium containing the ^{3}H-dThd.

After a 4 hour incubation at 37°, the specific radioactivity of the DNA in the slices was determined using a chemical extraction method based on the procedure described by Scott et al. (1956). The slices were homogenized in 1 N NaOH and incubated at 37° for 1 hour. After centrifugation to remove insoluble material, a 1 ml aliquot of the clear supernatant was acidified with 0.2 ml of 6 N HCl to give a final pH of approximately 2.0. The precipitated protein and nucleic acid (mainly DNA) was washed twice with 1 N NaCl, pH 2.0,

and finally the precipitate was digested with 1 N $HClO_4$ for 20 min at 60°. An aliquot of the clear supernatant was taken for radioactivity measurement by scintillation spectroscopy and the absorbance at 265 nm was measured. The specific radioactivity was calculated as ^{3}H-dpm per microgram of DNA, assuming a concentration of 50 µg of DNA per ml to give an absorbance of 1.0 at 265 nm.

DNA replicative synthesis was expressed as the specific radioactivity of the cellular DNA at the end of a 4 hour incubation period in the absence of HU. DNA repair synthesis was calculated by subtracting the specific radioactivity of DNA of tissue slices from control animals from that of carcinogen-treated animals when the slices were incubated with both ^{3}H-dThd and HU.

Measurement of DNA Replication and Repair Synthesis in Lymphocytes from Spleen or Blood

Blood from experimental animals was collected into heparinized syringes and layered onto Ficoll-Hypaque gradients according to published methods (Guirgis et al., 1978). After centrifugation, the lymphocyte-containing layer was collected and the cells washed twice with Dulbecco's minimal essential medium (DMEM, Grand Island Biological Co.). The spleens of experimental animals were gently homogenized to disperse the cells and the resulting suspension layered on Ficol-Hypaque gradients as in the case of the blood lymphocytes.

The washed cell suspensions were dispensed into wells of Microtiter plates (Falcon Plastics, Oxnard, CA) at 1-2 x 10^5 cells per well, depending on the experiment. Viability of the cells was determined by the trypan blue dye exclusion method (Phillips, 1973). For measurement of DNA replicative synthesis, the wells contained ^{3}H-dThd (5 µCi/ml); for measurement of DNA repair synthesis, the wells also contained 10^{-2}M HU. After 1.5 or 3 hours incubation at 37°, the cells were harvested by collecting them onto glass fiber filters and washing exhaustively with distilled water. After drying, the radioactivity remaining with the filters was determined by scintillation counting, and calculated as ^{3}H-dpm per 10^5 cells. DNA repair synthesis was calculated by subtracting the values obtained with cells from control animals from the values obtained with carcinogen-treated animals.

Experimental Protocol

For this study, three carcinogens have been chosen which have somewhat different modes of action, and different organotropies. They are benzo(a)pyrene (BaP), which causes tumors at the site of injection or contact, 4-nitroquinoline-1-oxide (4NQO), which causes

lung tumors in rats and mice when given systemically, and dimethylnitrosamine (DMN), which causes liver tumors when injected. Reports of others (Stich and Kieser, 1974; Laishes, et al., 1975) indicate that 4NQO and DMN do induce a DNA repair response in the target organs for carcinogenesis, but in vivo/in vitro correlation studies have not been done. BaP will be used as an example of an environmental carcinogen. It produces lung tumors when given intratracheally, and it is in this mode that it will be used in the present study.

Animal Exposure

At this writing, the experiments planned with BaP have not been completed; however, they are expected to be underway before the end of the present contract year. BaP will be administered by intratracheal inoculation of a suspension of BaP in 0.2% gelatin in 0.9% NaCl in animals lightly anesthetized with Metofane (methoxyflurane). Methods for this procedure have been published (Saffiotti et al., 1968; Kouri et al., 1976). Dosages will be in the range of 50-500 micrograms per animal.

4NQO was administered sc as a solution in dimethylsulfoxide (DMSO) over a range of doses from 50-250 mg/kg bw as indicated in the Results section.

DMN was given as a solution in 0.9% NaCl over a dose range of 10-300 mg/kg bw as indicated.

Animal Species

The two species used were C57BL/6J male mice, 6-8 weeks old and Fischer 344 male rats, also 6-8 weeks old at the time of experimentation.

RESULTS

Studies with 4-Nitroquinoline-1-oxide (4NQO)

In Vitro Exposure of Mouse Tissues to 4NQO. An experiment was done in which lung slices from C57BL/6J mice were exposed in vitro to 4NQO at 4 concentrations (10^{-4}, 10^{-5}, 5×10^{-6}, 10^{-6} M). DNA repair synthesis was measured as described under Methods. The results, presented in Table 1, indicated that DNA repair was stimulated in the lung slices at all concentrations except 10^{-6}M. The optimum concentration appeared to be 10^{-5}M. A second experiment was done in which slices from liver, kidney, and spleen were used in addition to the lung slices. Only the kidney slices showed evidence of a DNA repair response at 10^{-5}M 4NQO. The results indicated that the lung was the most responsive of the organs tested.

TABLE 1. INDUCTION OF DNA REPAIR SYNTHESIS IN LUNG SLICES IN VITRO BY 4NQO

As described in the text, lung slices from male C57BL/6J mice were incubated for 4 hours in vitro with ^{3}H-dThd, HU, and 4NQO. The values are ^{3}H-dpm per microgram of DNA and are based on triplicate samples from each mouse. DNA repair synthesis was calculated by subtracting the incorporation of ^{3}H-dThd into control slices from that incorporated into the slices exposed to 4NQO

Experiment 1

Mouse	Untreated slices	10^{-6}M 4NQO	$5x10^{-6}$M 4NQO
A	187	265	509
B	251	277	559
C	296	273	584
Means:	245 ± 55	272 ± 6	551 ± 38
Calculated DNA Repair		27 ± 39	306 ± 47

Experiment 2

		10^{-5} 4NQO	10^{-4}M 4NQO
A	265	981	693
B	321	888	523
C	233	808	733
Means:	273 ± 45	892 ± 87	650 ± 112
Calculated DNA Repair		619 ± 69	377 ± 85

Induction of DNA Damage In Vivo in the Mouse by 4NQO. Groups of 3 mice were injected sc with 4NQO dissolved in DMSO as described in Materials and Methods. The doses employed were 10, 50, 100, 125, and 250 mg/kg bw. Controls were injected with DMSO only. One hour after treatment, the mice were killed with an overdose of sodium pentobarbital and DNA repair synthesis was measured as described. The results (Table 2) showed a dose response in the lung and the spleen lymphocytes. Data from other tissues is not available at this time except for the 50 mg/kg dose for which the response of liver and kidney was minimal.

Induction of DNA Damage In Vivo in the Rat by 4NQO. Preliminary results with rats have indicated that DNA damage is induced in vivo with 4NQO. Treatment with 50 mg 4NQO/kg bw followed by in vitro measurement of DNA repair synthesis as for the mice, indicated that DNA repair synthesis in the lung was about 1/3 as active as in the mice when the assay was done in the same way. Further studies with rats are pending.

TABLE 2. DNA REPAIR SYNTHESIS IN MOUSE TISSUES AS MEASURED IN VITRO ONE HOUR AFTER INJECTION OF THE MICE WITH 4NQO

DNA Repair synthesis was calculated by subtracting the incorporation of radioactivity into cells or tissue from control mice from the radioactivity incorporated into tissue from treated mice when hydroxyurea was present in both cases. The values for lung, liver, and kidney are ^{3}H-dpm per microgram of DNA. The values for spleen lymphocytes are ^{3}H-dpm per 2 x 10^{5} cells, ± S.D. The numbers in parentheses are the number of animals for each group.

Dose mg/kg	Lung	Liver	Kidney	Spleen
10 (3)	N.S.[a]	N.D.[b]	N.D.	N.S.
50 (3)	356 ± 114	29 ± 17	10 ± 14	71 ± 60
100 (3)	291 ± 57	N.D.	N.D.	259 ± 48
125 (2)	518 ± 32	N.D.	N.D.	551 ± 191
250 (3)	649 ± 133	N.D.	N.D.	567 ± 471

[a] N.S. = not significantly different from zero.
[b] N.D. = no data.

Studies with Dimethylnitrosamine (DMN)

In Vitro Treatment of Mouse Tissues with DMN. Lung, liver, and kidney slices from C57BL/6J male mice were incubated in vitro under conditions described in Methods with 10^{-3}M DMN. Control slices were incubated without DMN. The results (Table 3) indicate that both lung and liver DNA were damaged by the in vitro treatment with DMN and could undergo repair synthesis, while the kidney showed less of a response. No DNA repair was detected in spleen lymphocytes incubated in vitro with DMN.

TABLE 3. INDUCTION OF DNA REPAIR SYNTHESIS IN VITRO IN MOUSE TISSUES BY DMN

Lung, liver, and kidney slices and spleen lymphocytes were incubated in vitro with 10^{-3}M DMN, and DNA replication and repair synthesis were assayed as described in the text. The values for lung, liver and kidney are ^{3}H-dpm per microgram of DNA and for the spleen lymphocytes are ^{3}H-dThd per 2 x 10^{5} cells.

Tissue	Replicative Synthesis	DNA Repair Synthesis
Lung	1608 ± 82	324 ± 72
Kidney	151 ± 26	68 ± 32
Liver	220 ± 49	220 ± 13
Spleen Lymphcoytes	657 ± 113	Not Detected

Induction of DNA Damage In Vivo in the Mouse by DMN. C57BL/6J male mice were injected ip with DMN dissolved in 0.9% NaCl and one hour later selected tissues were removed and assayed for DNA repair synthesis as described under Methods. Table 4 presents the results of a dose-response study over the range of 3-300 mg DMN/kg bw. A dose-dependent response was seen for lung liver, and kidney, with the lung showing most active repair synthesis. DNA repair synthesis was detected in the peripheral and spleen lymphocytes only at the highest dose tested (300 mg/kg).

No studies with DMN have been done in the rat.

TABLE 4. DNA REPAIR SYNTHESIS IN THE MOUSE AFTER IN VIVO TREATMENT WITH DMN

DNA repair synthesis was calculated as in Table 2.

Dose	^{3}H dpm per microgram of DNA				
mg/kg	Lung	Liver	Kidney	lymphocytes	Spleen
3	100±28	N.S.[a]	N.D.[b]	--	--
30	519±99	60±20	25±13	N.S.	N.S.
75	822±38	82±64	43±16		
150	707±240	208±144	65±26	N.S.	N.S.
300	715±77	174±51	98±33	186±66[c]	74±40[c]

[a] N.S. = not significantly different from zero.
[b] N.D. = no data.
[c] Values for peripheral lymphocytes and spleen are ^{3}H-dpm per 2 x 10^5 cells.

DISCUSSION AND CONCLUSIONS

The studies conducted thus far with mice support the contention that chemical carcinogens damage DNA in the target organ for carcinogenesis. The data showed substantial variation among the organs studied, with the lung being the one most active in DNA repair synthesis after in vivo or in vitro exposure to the chemicals tested.

One goal of this study was to determine whether lymphocytes might be useful as an indicator population for carcinogen exposure. In the studies with 4NQO, it was found that exposure of spleen lymphocytes to 4NQO in vitro produced only a minimal DNA repair response, but assay of DNA repair synthesis in spleen lymphocytes from mice exposed to 4NQO in vivo showed a very marked response (Table 2). On the other hand, DMN produced no significant DNA repair synthesis in spleen lmphocytes, and a response in peripheral lymphocytes only at the highest dose tested in vivo, 300 mg/kg bw. The results with 4NQO

suggest that metabolic activation of this chemical occurs in vivo and stimulated the DNA repair response in the spleen. The spleen lymphocytes themselves may not contain the enzymes necessary to convert 4NQO to its active form. We have not assayed the mouse tissues for the presence of the required enzymes.

EFFECT OF ACUTE HYDRAZINE EXPOSURE ON CELLULAR PROLIFERATION, DNA REPLICATION, AND DNA REPAIR IN RODENT LUNG (THRU EXPT. #559)

INTRODUCTION

A variety of chemicals, including potential environmental pollutants, have been shown to cause damage to the lung when inhaled or when given systemically to experimental animals. These include oxidant gases (NO_2, O_3), hydrocarbons (e.g., naphthalene, halogenated benzene), naturally-occuring toxins (patulin, 4-ipomeanol) and tobacco smoke.

Previous studies of mice exposed to relatively low levels of hydrazine did not show significant differences among control and exposed groups in regard to lung DNA replication, DNA repair capacity or cellular proliferation. However, in the studies reported here, the animals were exposed to higher levels of hydrazine (750 ppm).

Studies in this laboratory with certain lung toxins have shown both short- and long-term effects in the lungs of rats and mice. For example, butylated hydroxytoluene (BHT) when given ip to mice caused type 1 cell damage and induced the proliferation of their precursor type 2 cell. However, after 3-4 weekly injections, the mice no longer responded to BHT with a burst of cellular proliferation, but appeared to be completely resistant to the effects of BHT. On the other hand, cellular proliferation in the lungs of mice chronically exposed to cigarette smoke remained at a level 2-3 fold higher than controls as long as smoke exposure was continued (Rasmussen et al., 1981).

In the acute hydrazine exposure studies done at the Toxic Hazards Research Unit, AMRL, WPAFB, Ohio (THRU), some cellular damage to the respiratory tract was expected. The high solubility of hydrazine in aqueous solvents suggested that a large fraction would be absorbed in the nasal turbinates and upper airways of the test rodents and that most cellular effects would be produced in these areas. However, because of the high levels of hydrazine to be used, some could penetrate to the deeper lung, and there cause both cellular and biochemical effects.

The objectives of the studies conducted at UC Irvine on the rats and hamsters exposed to hydrazine at the THRU were:

1. Determine the effect of a single exposure to hydrazine at 750 ppm for one hour on:

a. DNA replication in the lung
b. DNA repair capacity in the lung
c. Cellular proliferation in the lung and nasal turbinates
d. Metabolic conversion of ^{3}H-BaP to hydroxylated derivatives by lung slices.

2. Determine the effects of repeated exposure to hydrazine on the above functions.

3. Determine whether delayed effects of hydrazine appear several days following exposure.

The exposures to hydrazine were conducted at the THRU and the animals shipped by air to UC Irvine. The first group of exposed animals was received at UC Irvine in December, 1980. The animals had received a single one-hour exposure to hydrazine at 750 ppm and were examined at UC Irvine at about 48 hours postexposure. The results reported here are from this first group. Subsequent groups of animals were exposed 10 times before shipment, and an additional group was given a single exposure before shipment to UC Irvine. Because much of the analysis involves autoradiography, several weeks are required for exposure of the tissue sections before the analysis can be completed.

MATERIALS AND METHODS

Animal Exposures

The exposed animals were included among those exposed at the THRU as part of experiments which were intended to establish the toxic and carcinogenic hazard of repeated high level exposures. Details of exposure conditions may be found in periodic reports from the THRU.

Golden Syrian hamsters, male, 16 weeks old at the time of exposure, were used for the study. Eight each control and exposed were shipped to UC Irvine following a single exposure and eight each were shipped following 10 weekly exposures. Fischer 344 rats, male 11 weeks old at time of exposure, were exposed and shipped to UC Irvine as for the hamsters.

At the conclusion of the exposure at the THRU, the animals were held in a ventilated isolation chamber for 24 hours to permit thorough outgassing of hydrazine. The animals were then shipped by air to Los Angeles where they were met and transported immediately to

the laboratory at UC Irvine. The analyses described below were done, except as indicated, on the morning following arrival at UC Irvine.

Cellular Proliferation in Lung and Other Tissues

Cellular proliferation in the lung was estimated by two independent methods. They were 1) the incorporation of ^{3}H-dThd into cellular DNA as measured by a chemical method (Scott et al., 1956) and 2) the cellular labeling index in tissue sections from animals injected with ^{3}H-dThd. For the studies of labeling index in the lung, the animals were injected with ^{3}H-dThd (200 µCi/animal, 60 Ci/mmole) and killed one hour later by an overdose of sodium pentobarbital. The lungs were fixed in buffered 10% formalin (4% HCHO) by inflation via the trachea, and maintained inflated at a pressure head of 30 cm of water for 72 hours. The portion of the skull containing the nasal turbinates was cleaned of excess muscle and fat and fixed by immersion in 10% buffered formalin for at least 72 hours. The turbinates were decalcified by immersion for 3 weeks or more in 6% EDTA buffered to pH 7.6 (0.05M Tris) with weekly changes of the bath. The ratio of tissue/bath was about 1/500.

The tissues were embedded in paraffin and sectioned at 4-5 µm using conventional methods.

Autoradiographs were prepared by dipping the slides carrying the sections in Eastman NTB-2 liquid emulsion, diluted 1:1 with distilled water. After drying, the slides were stored at 4°C in light-tight boxes with desiccant until development with Kodak D-19. The slides were stained with H and E according to usual methods.

DNA Replication and Repair Synthesis in Lung

The methods employed were the same as those described in the previous section of this report. In brief, the lungs were inflated in situ with warm melted agar, then removed and freehand sections cut with a razor blade to a thickness of about 1 mm. These slices were then incubated in vitro with ^{3}H-dThd, HU, and MMS as described previously and in the table legends.

DNA Replication and Repair Synthesis in Spleen and Peripheral Blood Lymphocytes

The methods were the same as described in the previous section of this report. Briefly, whole heparinized blood or dispersed spleen cells were layered on Ficoll-Hypaque gradients and the lymphocytes collected following centrifugation. The cells were incubated with the medium appropriate to the experiment in wells of Falcon microtiter plates with 1-2 x 10^{5} cells per well. Incorporation of

^{3}H-dThd was measured by collecting the cells on glass fiber filters and after washing extensively with water; the radioactivity was measured by scintillation counting.

^{3}H-Benzo(a)pyrene Metabolism by Lung Slices

Lung slices prepared as described above were incubated in medium containing ^{3}H-benzo(a)pyrene (^{3}H-BaP, Amersham). (The structure and numbering scheme for BaP are shown in Figure 1.) The incubation medium consisted of Dulbecco's phosphate buffered saline (pH 7.2-7.4) supplemented with F12K amino acids (Kaighn, 1973) and 1% fetal bovine serum (Grand Island Biologicals, Inc.) to which was added ^{3}H-BaP (10 μCi/μg) dissolved in acetone. Lung slices equivalent to approximately 100-150 mg wet weight of tissue were incubated in a total volume of 2 ml containing 20 μCi of ^{3}H-BaP. At the end of a 4 hour incubation period at 37°, the tissue was homogenized in the incubation medium and the mixture extracted twice with 4 ml of ethyl acetate. The organic extracts were pooled, taken to near dryness under N_2, and subjected to thin layer chromatography (TLC) on Mylar-backed silica gel plates using benzene and benzene:ethanol (19:1) sequentially as solvents. Authentic nonradioactive derivatives of BaP were added to the extracted metabolites to serve as markers on the TLC plates. The standard compounds were obtained from the National Cancer Institute. The locations of the metabolites were determined by the fluorescence of the standards and scanning of the TLC plates with a radiochromatogram scanner. Finally, the metabolites were quantified by cutting the TLC plates into segments carrying the individual metabolites, and radioactivity measured using a scintillation spectrometer. Radioactivity not extracted from the incubation mixture was measured by scintillation counting of an aliquot of the aqueous phase of the extraction mixture.

RESULTS

DNA Replication and Repair Synthesis in Lungs of Rats and Hamsters Exposed to Hydrazine

The first group of animals was exposed at the THRU on December 8, 1980 and shipped by air to UC Irvine on December 9. One-half of the group was analyzed the following day as described under Methods.

Upon arrival at Los Angeles International airport, on the evening of December 9, the animals were housed in an unheated warehouse for approximately 2 hours at a temperature of about 10°C. All of the hydrazine exposed animals appeared definitely sick with ruffled fur, hunched back and possible photophobia in the hamsters.

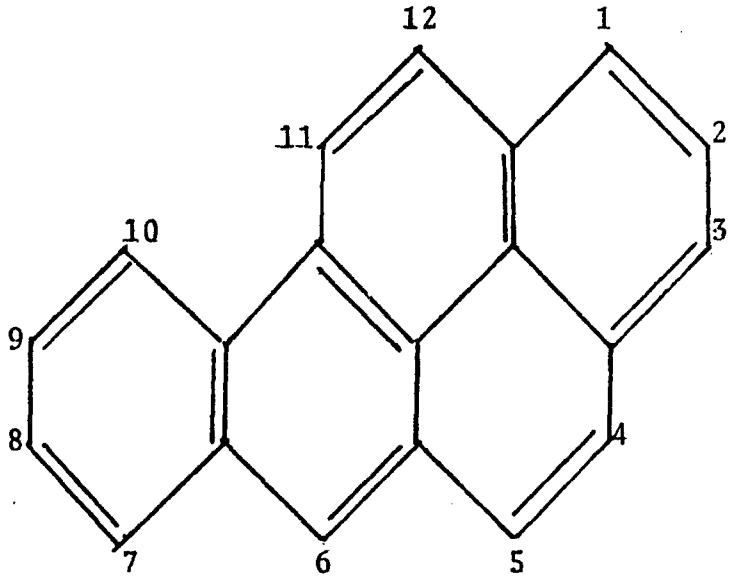

Figure 1. Benzo(a)pyrene numbering scheme.

Studies of DNA replication and DNA repair synthesis in the lungs was begun on the morning of December 10, and a second group was studied on December 15. The results obtained with the first shipment are shown in Table 5.

Both rats and hamsters showed a decrease in DNA replication of about 50% as measured in the lung slices. At 7 days postexposure, DNA replication in the rat lung slices was several fold greater than that seen at 2 days, but DNA repair synthesis in response to MMS-induced damage was only slightly increased (20-65%). DNA replication in the hamster lung slices was also elevated at 7 days (2-5 fold) as was DNA repair synthesis (16-98%).

The above experiment was repeated in May 1981, and the results are shown in Table 6. In this experiment, no significant difference was seen between the results with hydrazine-exposed animals and the controls. Because of scheduling constraints, the animals could not be assayed until 4 days after the hydrazine treatment. At 7 days post-treatment, DNA replication was increased in the rat lung slices,

TABLE 5. DNA REPLICATION AND REPAIR SYNTHESIS IN LUNG SLICES FROM RATS AND HAMSTERS EXPOSED ONCE TO HYDRAZINE AT 750 PPM FOR ONE HOUR ON 12/8/80

Values are ^{3}H-dpm per microgram of DNA and are based on triplicate samples from each animal.

	Assayed at 2 Days Post-Hydrazine		Assayed at 7 Days Post-Hydrazine	
	Replicative DNA synthesis	DNA repair synthesis	Replicative DNA synthesis	DNA repair synthesis
Rats:				
Control #1	687±54	417±44	4316±287	685±11
Control #2	637±51	410±26	5304±521	518±40
Hz #1	334±33	397±15	2802±126	489±57
Hz #2	351±12	430±27	4004±347	521±22
Hamsters:				
Control #1	3362±141	1093±68	6747±1132	1303±133
Control #2	2726±141	881±265	5867±495	1019±66
Hz #1	1478±96	688±81	7698±536	1365±94
Hz #2	1536±68	880±22	5754±191	1239±70

but decreased in the hamster lung slices. There were no differences in DNA repair synthesis among any of the groups.

Animals exposed 10 times to hydrazine were also received in May 1981, and DNA replication and repair synthesis in lung slices were measured as described. In these animals also, there was no apparent effect of the hydrazine treatment (Table 7). However, again, the level of DNA replication was higher in the lungs of the animals assayed at 7 days post-treatment compared to those assayed at 4 days. This increase in DNA replication has now been seen in all three groups of animals which have been sent to UC Irvine as part of this experiment. Comparison of the data with that obtained in previous similar studies with rats and hamsters suggested that the level of DNA replication in the animals studied at 4 days was depressed, rather than the alternative that DNA replication was stimulated in the animals studied at 7 days, and that a recovery to normal levels occurred in the latter animals during their holding at UC Irvine prior to study. Although specific experiments have not been done, we speculate that the depressed DNA replication in the animals assayed at 4 days was due to the stressful effects of the air shipment and related suboptimal housing conditions of the animals.

TABLE 6. DNA REPLICATION AND REPAIR SYNTHESIS IN LUNG SLICES FROM RATS AND HAMSTERS EXPOSED ONCE TO HYDRAZINE AT 750 PPM FOR ONE HOUR ON 5/15/81

Values are ^{3}H-dpm per microgram of DNA and are based on triplicate samples from each animal, except as indicated.

	Assayed at 4 Days Post-Hydrazine		Assayed at 7 Days Post-Hydrazine	
	Replicative DNA Synthesis	DNA Repair Synthesis	Replicative DNA Synthesis	DNA Repair Synthesis
Rats:				
Control #1	639 ± 48	394 ± 9	#3 1499 ± 342	370 ± 25
Control #2	635 ± 36	401 ± 28	#4 1325 ± 81	353 ± 36
Hz #1	733 ± 2	412 ± 65	#3 1540 ± 143	234 ± 69
Hz #2	757 ± 178	383 ± 25	#4 998 ± 82	534 ± 43
Hamsters:				
Control #1	1872 ± 17	538 ± 159	#3 1024 ± 140	458 ± 230
Control #2	1887 ± 188	595 ± 69	#4 1718 ± 198	469 ± 91
Hz #1	2111 ± 58	603 ± 98	#3 1794 ± 343	420 ± 184
Hz #2	3238 ± 201	879 ± 88	#4 2134 ± 123	399 ± 36[a]

[a] n = 2 for this value

Preliminary examination of autoradiographs of lung sections from the animals received December 8, 1980, indicated no great effect of the hydrazine exposure. The sections of rat lung from animals assayed at 48 hours post-treatment showed a normal appearing lung. There was a slight sloughing of bronchiolar epithelial cells in the terminal bronchioles, but no other obvious evidence of damage. Both control and treated animals showed minimal perivascular and peribronchiolar lymphocyte infiltration. The ^{3}H-thymidine labeling index in the rats was less than 0.001 for both the controls and treated. The picture in the hamster lungs was similar, except that the ^{3}H-thymidine labeling index was somewhat higher (0.010-0.012), in agreement with the finding of increased ^{3}H-dThd incorporation into lung slices in vitro (Table 5).

Autoradiographic studies of the rats and hamsters exposed 10 times to hydrazine are in progress. Lungs from these animals have been fixed by inflation with buffered 10% formalin at 30 cm of water pressure, and embedded in plastic for semithin sectioning. This latter procedure will permit morphometric measurements and offers the possibility of ultrastructural examination if indicated after preliminary examination by light microscopy.

TABLE 7. DNA REPLICATION AND REPAIR SYNTHESIS IN LUNG SLICES FROM RATS AND HAMSTERS EXPOSED 10 TIMES TO HYDRAZINE AT 750 PPM

Values are ^{3}H-dpm per microgram of DNA and are based on triplicate samples from each animal.

	Assayed at 4 Days Post-Hydrazine			Assayed at 7 Days Post-Hydrazine	
	Replicative DNA Synthesis	DNA Repair Synthesis		Replicative DNA Synthesis	DNA Repair Synthesis
Rats:					
Control #1	435±20	400±16	#3	875±101	392±37
Control #2	398±16	301±30	#4	819±67	428±49
Hz #1	577±14	317±32	#3	686±32	358±43
Hz #2	455±16	350±24	#4	2105±143	320±52
Hamsters:					
Control #1	1553±84	698±62	#3	1863±122	609±46
Control #2	1133±85	520±27	#4	2183±256	655±15
Hz #1	1619±45	634±11	#3	2530±601	638±17
Hz #2	1498±41	517±28	#4	2774±229	921±51

^{3}H-BaP Metabolism by Lung Slices from Hydrazine Exposed Rats and Hamsters

Hamster lung slices from all treatment groups showed very low enzymatic activity in the conversion of ^{3}H-BaP to polar metabolites. There was no detectable difference between treated and controls in any of the groups. The results, shown in Tables 8-11, indicated that hydrazine treatment did not increase the level of BaP metabolism, i.e., hydrazine did not induce or activate lung P-450 enzymes concerned with BaP metabolism.

The rat lung slices were substantially more active than the hamster slices in metabolism of BaP. In the group of animals received in December, 1980, which had been exposed one time to 750 ppm of hydrazine, there was a clear difference between the treated and control rats in those animals assayed at 48 hours after the hydrazine exposure. The overall level of ^{3}H-BaP metabolism was about 3-fold lower in the exposed animals (Table 8) compared to the controls. This difference was not seen in the rats assayed 7 days after the hydrazine exposure. Examination of the metabolite profiles indicated that the difference was due to a reduced production of all metabolites, but the monohydroxylated derivates were reduced to the

TABLE 8. METABOLISM OF ^{3}H-BaP BY LUNG SLICES FROM RATS AND HAMSTERS EXPOSED ONCE TO HYDRAZINE AT 750 PPM ON 12/8/80

Lung slices were prepared and incubated with ^{3}H-BaP as described in the text. Values are the percentage of input ^{3}H-BaP converted to hydroxylated produced during the 4 hour incubation period. The blank value from medium incubated without tissue has been subtracted the the data.

	2 Days post-hydrazine			7 Days post-hydrazine		
Blank:	1.37			Blank:	1.25	
Rats:			Average			Average
Control	#1A	5.86	5.23	#3A	2.31	2.50
	#1B	4.60		#3B	2.70	
Control	#2A	3.18	3.48	#4A	1.85	2.36
	#2B	3.78		#4B	2.86	
Hz	#1A	1.63	1.60	#3A	3.67	3.41
	#1B	1.56		#3B	3.15	
Hz	#2A	1.43	1.28	#4A	2.79	2.48
	#2B	1.12		#4B	2.16	
Hamsters:						
Control	#1A	0.99	0.96	#3A	(-0.08)[a]	0.0
	#1B	0.94		#3B	0.02	
Control	#2A	0.50	0.36	#4A	0.21	0.04
	#2B	0.23		#4B	(-0.17)[a]	
Hz	#1A	0.81	0.55	#3A	0.04	0.0
	#1B	0.29		#3B	(-0.04)[a]	
Hz	#2A	0.28	0.52	#4A	0.16	0.11
	#2B	0.77		#4B	(-0.05)[a]	

[a] The yields of metabolites from these samples, when calculated by this method, appeared to be lower than the blank value. However, metabolic activity was present as indicated by the presence of specific metabolites on TLC analysis.

greatest extent (Table 9). In the groups of animals received in May 1981, no difference in ^{3}H-BaP metabolism was seen between any of the groups. However, the earliest time of assay of the hydrazine exposed animals was at 4 days after the exposure (Table 10) These results suggest that a toxic effect may be produced immediately following hydrazine exposure, but the animals recover in a few days, and show normal levels of enzymatic activity toward BaP. Metabolism of ^{3}H-BaP by lung slices from animals exposed 10 times to hydrazine was not different from controls (Table 11), indicating no cumulative effect

of the exposures, but again the earliest assay was 4 days after the last hydrazine treatment.

TABLE 9. PRODUCTION OF SPECIFIC METABOLITES OF ^{3}H-BaP BY LUNG SLICES FROM RATS EXPOSED ONCE TO HYDRAZINE AT 750 PPM ON 12/8/80

Lung slices were prepared and incubated with ^{3}H-BaP as described in the text. The values are pmoles of metabolite produced per mg total protein present during the 4 hour in vitro incubation period, and are the average of duplicate samples.

	Assayed at 2 days post-hydrazine			
Treatment	9,10-Diol[a]	7,8-Diol	4,5-Diol	BP-OH[b]
Control #1	34.5	28	15	198
Control #2	25.5	20	10	94.5
Hydrazine #1	16.5	10.5	4.5	33.0
Hydrazine #2	17.5	11.0	8.0	46

	Assayed at 7 days post-hydrazine			
Treatment	9,10-Diol	7,8-Diol	4,5-Diol	BP-OH
Control #1	19.5	25.5	9.5	118
Control #2	19.5	13.5	7.0	84.5
Hydrazine #1	24.0	25.0	10.5	126
Hydrazine #2	21.5	20.0	9.5	114

[a] Diol = dihydrodihydroxy BaP.
[b] BP-OH = BaP monohydroxylated derivatives, including predominantly 3-OH-BaP and 9-OH-BaP.

TABLE 10. METABOLISM OF ^{3}H-BaP BY LUNG SLICES FROM HYDRAZINE-EXPOSED RATS AND HAMSTERS

Lung slices were incubated with ^{3}H-BaP as described in the text. The values are the percentage of input ^{3}H-BaP converted to more polar hydroxylated metabolites during the 4-hour incubation period. The blank value which has been subtracted from the experimental values was obtained by incubating ^{3}H-BaP for 4 hours in the same medium used for the lung slices, and performing the extraction as for the lung slices. The animals had been exposed once to hydrazine at 750 ppm. Duplicate samples were prepared from each animal.

	4 Days post-treatment			7 Days post-treatment			
			Average				Average
Hamsters:							
Hz	#1A	2.47	1.92	Hz	#3A	0.74	0.56
	#1B	3.01			#3B	0.38	
Hz	#2A	0.90	1.15	Hz	#4A	0.52	0.58
	#2B	1.40			#4B	0.63	
Control	#1A	0.28	0.24	Control	#3A	0.41	0.42
	#1B	0.21			#3B	0.43	
Control	#2A	1.21	1.06	Control	#4A	0.58	0.74
	#2B	0.92			#4B	0.91	
Blank 1.64				Blank 0.89			
Rats:							
Hz	#1A	12.19	11.94	Hz	#3A	3.28	3.51
	#1B	11.68			#3B	3.74	
Hz	#2A	5.93	4.67	Hz	#4A	3.72	4.19
	#2B	3.41			#4B	4.66	
Control	#1A	3.29	3.52	Control	#3A	3.31	4.38
	#1B	3.76			#3B	5.44	
Control	#2A	4.27	3.96	Control	#4A	3.16	2.87
	#2B	3.67			#4B	2.58	
Blank 1.64				Blank 0.89			

TABLE 11. METABOLISM OF ^{3}H-BaP BY LUNG SLICES FROM RATS AND HAMSTERS EXPOSED 10 TIMES TO HYDRAZINE AT 750 PPM

Lung slices were incubated with ^{3}H-BaP as described in the text. Values are the percentage of input ^{3}H-BaP converted to hydroxylated metabolites during the 4 hour incubation period. The blank value has been subtracted from the values.

		4 Days post-hydrazine			7 Days post-hydrazine	
Hamsters:			Average			Average
Hz	#1A	0.60	0.42	#3A	0.23	0.26
	#1B	0.24		#3B	0.30	
Hz	#2A	(-.08)	(0.0)	#4A	0.30	0.55
	2B	0.04		4B	0.80	
Control	1A	0.37	0.36	#3A	0.46	0.58
	1B	0.35		#3B	0.70	
Control	#2A	(-.04)	0.05	#4A	0.37	0.46
	#2B	0.09		#4B	0.55	
Rats:						
Hz	#1A	2.29	2.42	#3A	1.99	2.03
	#1B	2.56		#3B	2.07	
Hz	#2A	3.23	2.84	#4A	2.21	2.21
	#2B	2.46		#4B	No sample	
Control	#1A	3.30	3.08	#3A	1.34	1.58
	#1B	2.85		#3B	1.83	
Control	#2A	2.06	1.94	#4A	1.23	1.46
	#2B	1.82		#4B	1.70	

Cellular Effects of Hydrazine in the Nasal Turbinate Region

Hydrazine has been shown to induce tumors in the nasal turbinate region in rats possibly arising from cells of the olfactory epithelium, and therefore this tissue is of special interest. Autoradiographs were prepared from transverse sections of the turbinate regions at a point such that samples of the 3 major epithelial cell types would appear in the section. Figure 2 is a sketch of a sagittal section through the head of a rat. The regions marked "A", "B", and "C", are the approximate areas containing, respectively, stratified squamous epithelium, respiratory epithelium, and olfactory epithelium. The olfactory bulb of the brain is at "0". Transverse sections were cut at the dashed line marked "X". The usual appearance of the sections is shown in Figure 3, which indicates the major features of the turbinate region. (Only one side of the head is shown.) The locations of the epithelial cell types

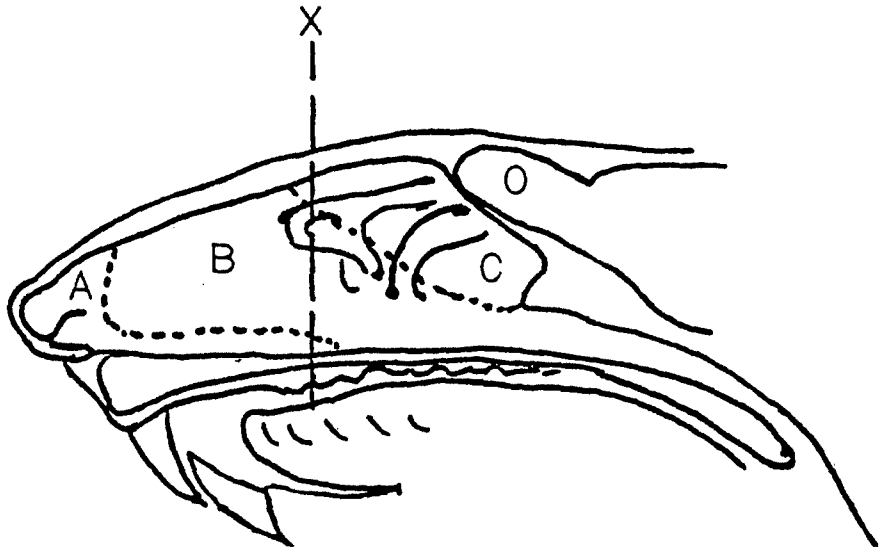

Figure 2. Sketch of sagittal section of rat head showing approximate locations of various epithelial types. A, stratified squamous epithelium; B, Respiratory epithelium; C, Olfactory epithelium; O, Olfactory bulb; X, Plane of section taken for analysis (After Hebel and Stromberg, 1976).

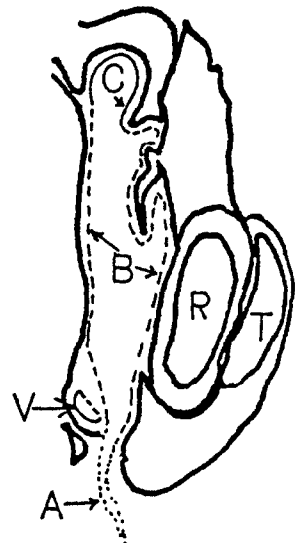

Figure 3. Sketch of transverse section of rat head showing relation ships of epithelial types at section "x" of Figure 2. A, Stratified squamous epithelium; B, Respiratory epithelium; C, Olfactory epithelium; R, Root of incisor; T, Lacrimal duct; V, Vomeronasal organ. Only one side of head is shown. (After Hebel and Stromberg, 1976).

are indicated as in Figure 2. Other structures are at "V", the vomeronasal organ; at "R", the root of the incisor, at "T", the lacrimal duct. The anatomical arrangement of the hamster is similar.

The effect of the hydrazine exposure in the rat was most marked in the respiratory epithelium. Controls showed well differentiated pseudostratified columnar epithelium or lower cuboidal ciliated epithelium throughout the expected turbinate region. In the exposed rats killed 48 hours postexposure, this epithelium was largely destroyed, and replaced by squamous or low cuboidal epithelium in which many labeled cells were seen (Figures 4 and 5). In the controls, only an occasional labeled basal cell was seen in this region (less than 1 labeled cell per 1000), but in the exposed rats, the labeling index was 0.20 in the squamous regions and 0.06 in the regions showing low cuboidal cells. In the rats killed at 7 days post exposure, the respiratory epithelium appeared nearly normal, indicating that repair of the hydrazine induced cellular damage was rapid and complete.

The olfactory epithelium was also affected in the rats, but the nature of the damage is not clear. The controls showed a layer of cells 6-10 cells thick, with uniform nuclei and no labeled cells (Figure 6). In the rats killed at 48 hours, the epithelial layer was still present, but the morphology of the cells was altered (Figure 7). The nuclei were not uniform in size or shape, and there were small darkly-staining bodies which may have been pyknotic nuclei. At 7 days post-treatment, the treated and controls were not different.

The stratified squamous epithelium at the entrance to the nasal cavity appeared to be unaffected by the hydrazine exposure, either in morphology or ^{3}H-dThd labeling index.

The damage caused by hydrazine to the nasal epithelium was more severe in the hamsters than in the rats. In the animals killed at 48 hours post-treatment, the stratified squamous epithelium at the entrance to the nasal cavity, was as in the rats, unaffected, but both the respiratory and olfactory epithelium were almost completely destroyed (Figures 8-11). The airways were filled with masses of sloughed cells and infiltrated with small lymphocytes and polymorphonuclear leukocytes. In the hamsters killed at 7 days post-treatment, the respiratory epithelium was essentially restored to its normal condition, and appeared the same as the control animals. The olfactory epithelium, however, had not been restored at the end of 7 days, and appeared as a layer of cuboidal cells, 1-5 cells thick with a ^{3}H-dThd labeling index of about 0.14 (Figure 12). No similar effects were seen in the other major structures of the nasal region,

Figure 4. Respiratory epithelium of control rat. Note pseudostratified columnar epithelium. Bar is 20 µm.

Figure 5. Respiratory epithelium of rat 48 hours post hydrazine exposure. Columnar epithelium is missing. Airway contains sloughed cells and leukocytes. Bar is 20 µm.

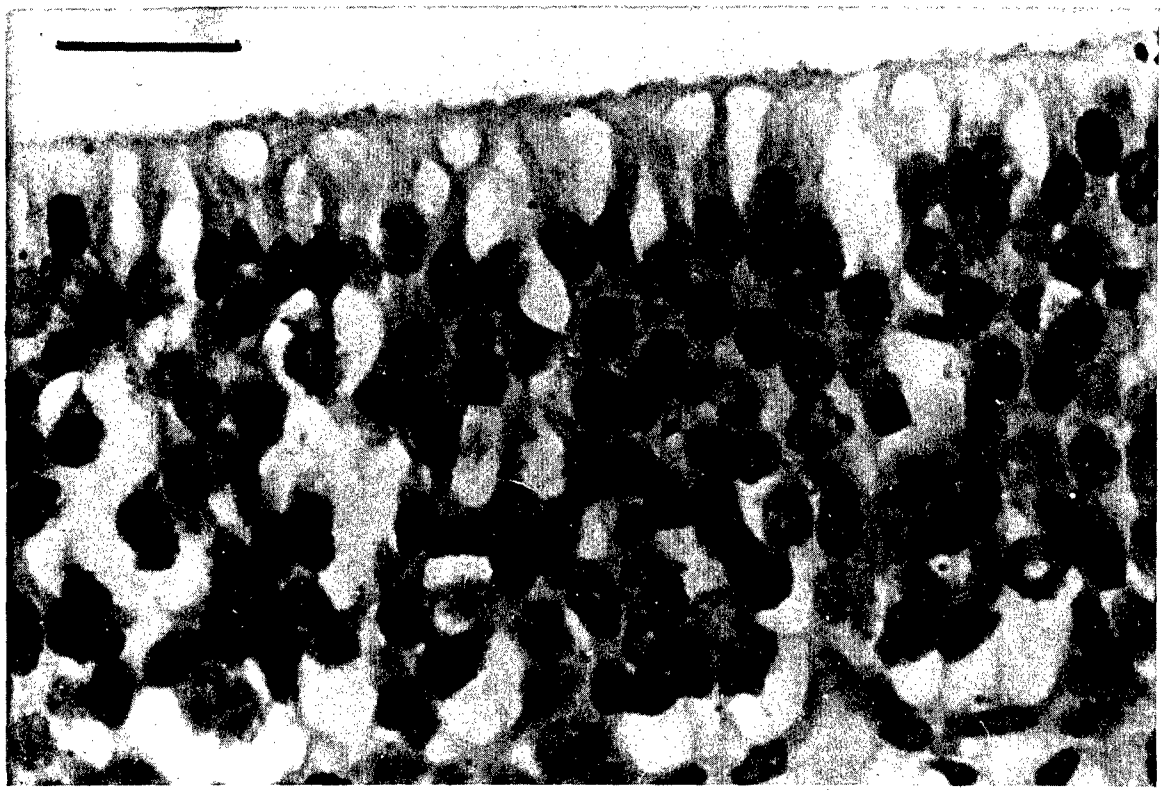

Figure 6. Olfactory epithelium of control rat. Note uniform nuclei. Bar is 20 μm.

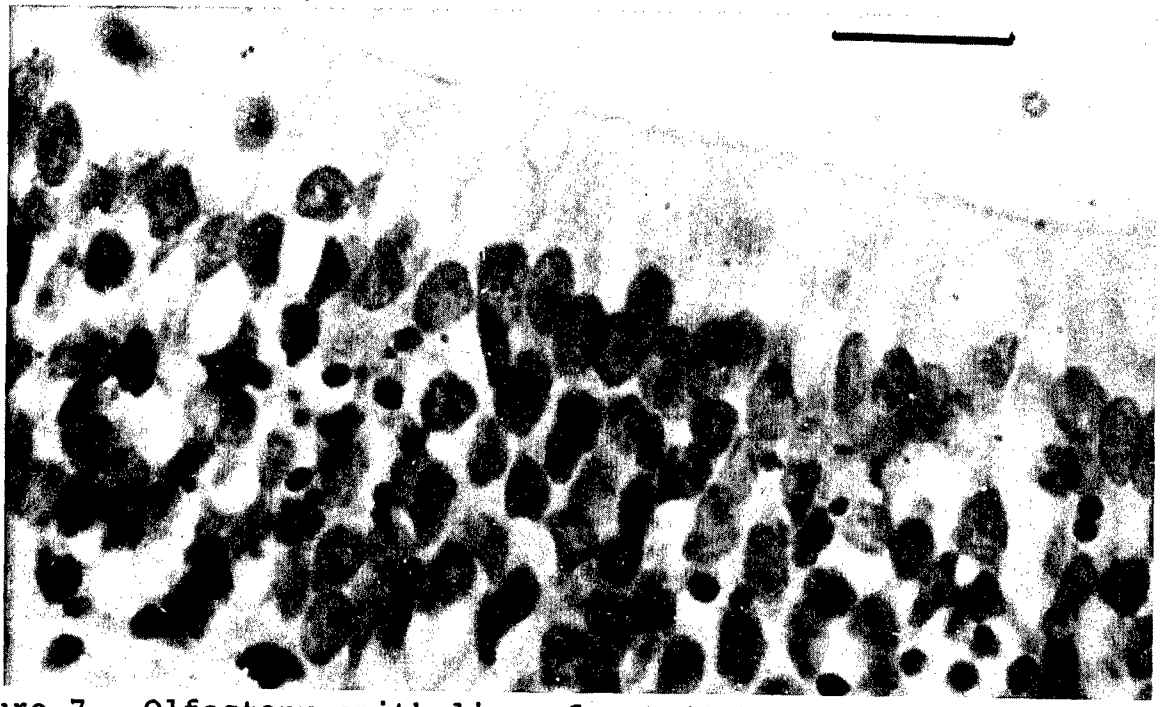

Figure 7. Olfactory epithelium of rat 48 hours post hydrazine. Note varying size nuclei, densely-staining objects. Bar is 20 μm.

Figure 8. Respiratory epithelium of control hamster. Note pseudostratified columnar epithelium and labeled cells. Bar is 20 μm.

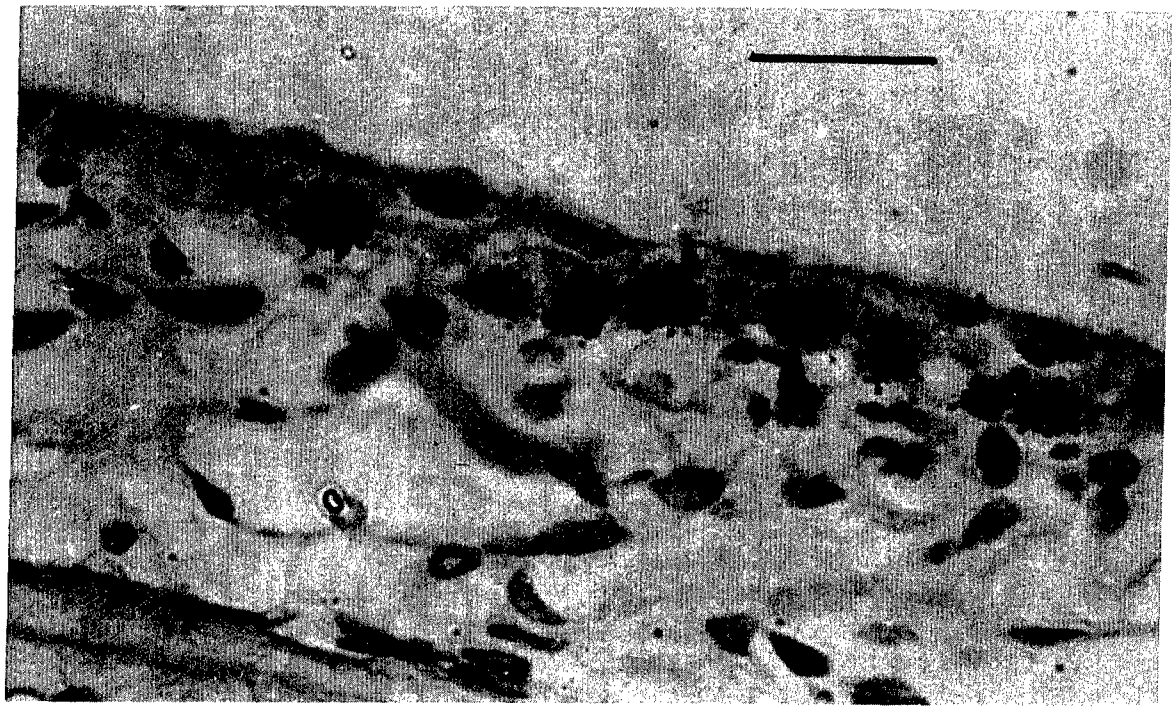

Figure 9. Respiratory epithelium of hydrazine exposed hamster, 48 hours post-treatment. Note loss of columnar epithelium and presence of many labeled cells. Bar is 20 μm.

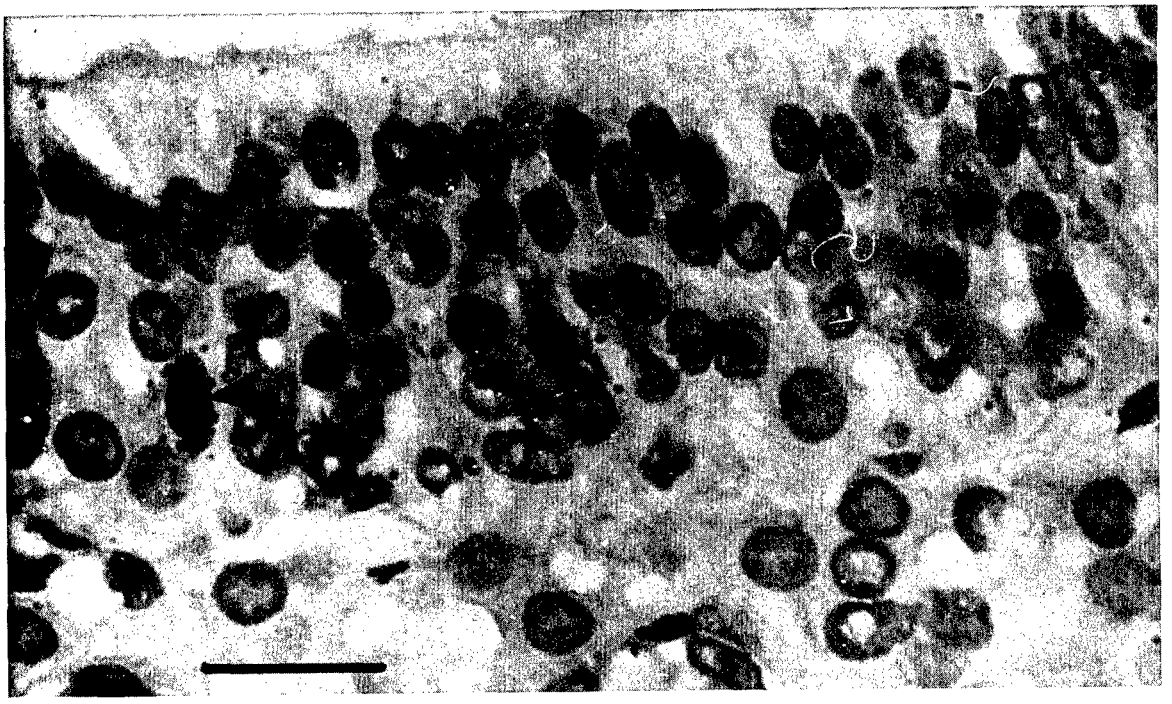

Figure 10. Olfactory epithelium of control hamster. Note columnar nature, with differentiated cells, and one labeled cell (arrow). Bar is 20 μm.

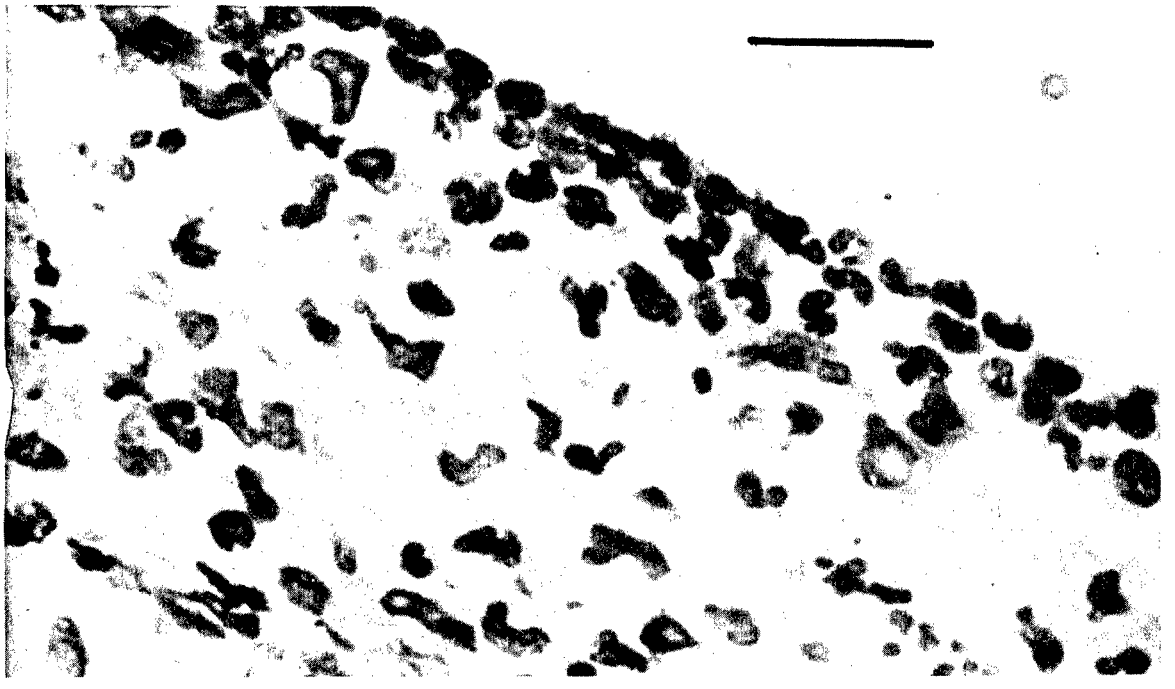

Figure 11. Olfactory epithelium of hamster exposed 48 hours previously to hydrazine. Note complete loss of columnar stucture. Cells have sloughed into the nasal cavity. Bar is 20 μm.

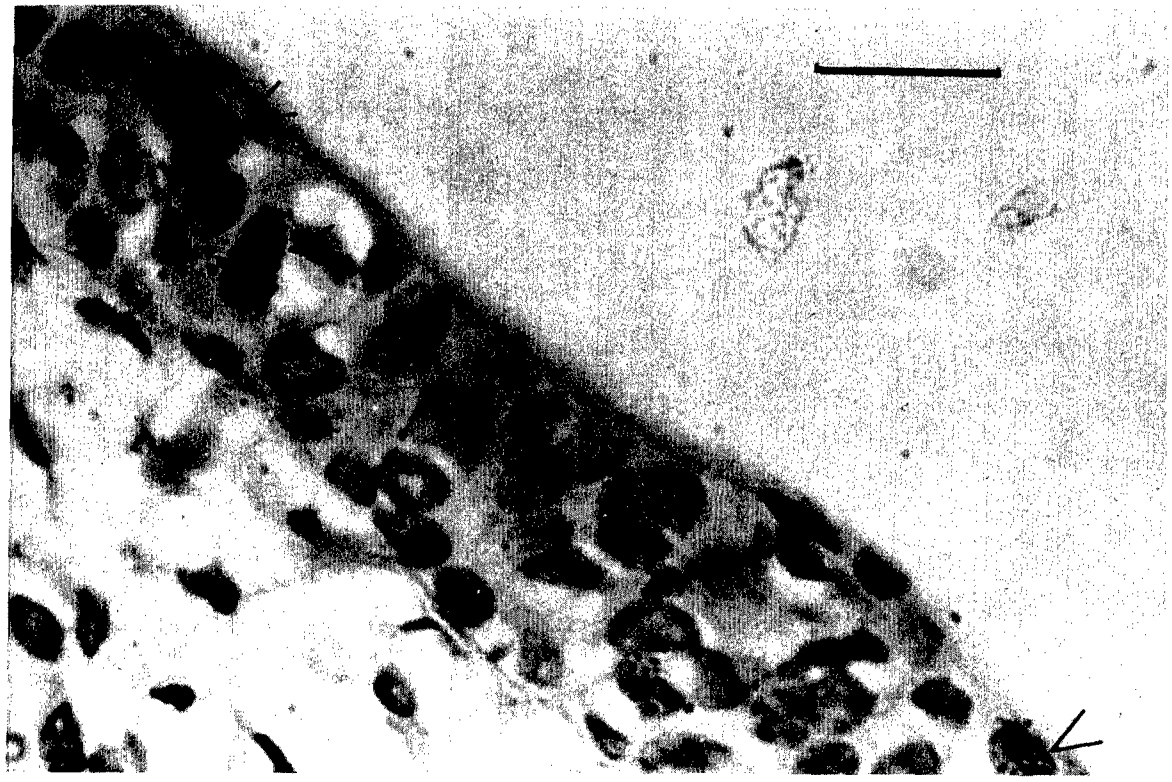

Figure 12. Olfactory epithelium of hamster at 7 days post hydrazine exposure. Columnar structure is not restored, but many labeled cells are present (arrows). Bar is 20 μm.

i.e., vomeronasal organ, lacrimal duct, which were present in the sections available for examination.

DISCUSSION AND CONCLUSIONS

Acute, high level exposure of rats and hamsters to hydrazine vapor appears to produce transient toxic effects in the respiratory tract. The major cellular and tissue damage was seen in the nasal turbinates of the rats and hamsters as might have been expected from the irritant effects of hydrazine. The extent of damage was different between the two species, with the hamsters showing much greater loss of respiratory and olfactory epithelial cells. The oncogenic effect of hydrazine has been shown in the rat, but the hamster, which suffers certainly equal, and probably more severe cellular effects may also prove to be susceptible.

Evidence that hydrazine vapors caused effects in the lungs of the rats was demonstrated by the experiments involving incorporation of ^{3}H-dThd into cellular DNA of lung slices, and those which measured the enzymatic conversion of ^{3}H-BaP to more polar derivatives. In both cases, the lung slices from hydrazine-exposed rats showed decreased activity when assayed at 48 hours after hydrazine exposure (Tables 5 and 8). The hydrazine-exposed hamsters showed a reduced level of ^{3}H-dThd incorporation at 48 hours, but not at later times (Table 5). The hamster lung slices from either controls or exposed animals had negligible enzymatic activity toward ^{3}H-BaP. This latter finding is consistent with previously published work (Wang et al., 1974) which showed that the basal level of lung P-450 enzymes is very low, but treatment of the animals with inducers of the enzymes will produce an increase in activity within 48 hours.

Recovery of the rats from the effects of the hydrazine, as judged by the cellular and biochemical assays performed here, seemed to be essentially complete by 7 days postexposure. This was not true for the hamsters, in which case the olfactory epithelium had not been completely restored at 7 days (Figures 10 and 12) following a single exposure to hydrazine. The results of the experiments involving 10 weekly exposures, which will be forthcoming, should be especially interesting since the olfactory epithelium would presumably receive successive exposures to hydrazine before the epithelium could recover to its normal state.

The data presently available from the animals exposed 10 times to hydrazine indicate that ^{3}H-dThd incorporation and ^{3}H-BaP metabolism by lung slices were not affected in a progressive manner by the repeated exposures. The conclusion is limited by the fact that the first opportunity for analysis of the repeatedly-exposed animals was 4 days after the last exposure, whereas the effects noted above on ^{3}H-dThd incorporation and ^{3}H-BaP metabolism were seen in animals at 48 hours after exposure. Nevertheless, based on the data presently available, there appeared to be no cumulative effect of the 10 hydrazine exposures in either the rats or hamsters. As noted above, analysis of cellular effects in the nasal turbinates and lungs must await completion of autoradiographic processing, which is now underway.

FUTURE PLANS

As indicated above, autoradiographic studies of the nasal turbinates and lungs of animals exposed 10 times to hydrazine are underway, and these studies will extend at least until the end of the present contract year. Portions of the lungs have been embedded in plastic and semithin sections cut for examination by light

microscopy. If lung lesions are found associated with the hydrazine exposures, further studies at the subcellular level (electron microscope) may be proposed.

STUDIES IN A RAT LUNG TUMOR MODEL: CELLULAR BIOCHEMISTRY AND CYTOGENETICS

INTRODUCTION

The use of animal models for the estimation of environmental carcinogens usually relies on the appearance of tumors at some time after treatment of the test animals. In most cases, the intermediate changes between the initial treatment of the animals and the final appearance of the tumors are neglected. The studies undertaken in this project are directed toward obtaining information during the latent period before tumor appearance which may be related in some way to the tumorigenesis process. The work reported here is a part of a coordinated program involving the staff at the THRU (Toxic Hazards Research Unit, AMRL, WPAFB), Dr. J.D. MacEwen, and Dr. Hoda Guirgis of the UC Irvine faculty. The overall goal is to obtain a more clear understanding of the immunologic, biochemical, and cytogenetic changes which occur during tumorigenesis in an animal model. Correlations between alterations in these factors and eventual tumor formation may be found which will then be of great use in the evaluation of possible tumorigenic effects of materials which may be tested in the future.

During the current year, range-finding studies have been underway at the THRU to establish the intratracheal dosage of 3-methylcholanthrene (MCA) which is known to produce a high incidence of lung carcinomas in male Fischer 344 rats within the span of about one year. When the dose regimen has been determined, a larger group of rats will be treated and, at predetermined intervals, sample groups will be killed and examined for alterations in various biochemical or cytogenetic factors which may have resulted from the MCA treatment, and may be related to tumorigenesis. Living cells and tissues will be sent to UC Irvine for analysis, and the lungs will be examined at the THRU for pathologic changes. The entire program is expected to extend through 1982.

This report describes preliminary studies conducted at UC Irvine to establish the feasibility of analyzing cells and tissues shipped from the THRU in the manner to be followed during the tumorigenesis study scheduled to begin in early 1982.

A part of the studies to be done during this project involves the measurement of metabolic conversion of ^{3}H-benzo(a)pyrene (^{3}H-BaP) to

hydroxylated products by liver, lung, and kidney cytochrome P-450 enzymes. These enzymes are responsible for both the detoxification and the metabolic activation of environmental polycyclic hydrocarbon carcinogens such as BaP. Therefore, alteration of these enzymes by exposure of experimental animals to environmental agents may affect the animals' susceptibility to carcinogenesis.

MATERIALS AND METHODS

Preparation and Shipment of Frozen Tissues

Rats were killed at the THRU with an overdose of sodium pentobarbital. The lungs, liver and kidney were removed and immediately frozen on solid CO_2, placed in plastic vials, and shipped to UC Irvine by air packed in solid CO_2. At UC Irvine, the tissues were stored either on solid CO_2 or in a -73° freezer.

Preparation of Microsomes and Analysis of ^{3}H-BaP Metabolism

The frozen tissues were thawed on ice and microsomes were prepared by homogenization of the tissues and fractional centrifugation according to published methods (Rasmussen and Wang, 1974). The final washed microsomal pellet was suspended in tris-sucrose buffer at a concentration of approximately 15 mg protein/ml.

Measurement of the conversion of ^{3}H-BaP to hydroxylated products by the microsomes was done in a manner similar to that used for the lung slices studies described above. The incubation mixtures contained 1.0 mg microsomal protein, 2.4 micromoles NADPH, and 10 μCi ^{3}H-BaP (2 μg) in a total volume of 2.0 ml of SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.4). After 30 min incubation at 37°, the reaction was stopped by chilling in ice and adding 4 ml of ethyl acetate. The mixture was vigorously agitated on a vortex mixer, centrifuged to separate the phases, and the upper organic phase removed. The ethyl acetate extraction was repeated, and the organic phase added to the first extract.

The metabolites of ^{3}H-BaP extracted into the ethyl acetate were analyzed by thin-layer chromatography (TLC) on silica gel plates using benzene and benzene:ethanol (19:1) sequentially as solvents. The locations of the metabolites were determined by a radiochromatogram scanner and by using authentic standard derivatives of BaP. The metabolites were quantified by cutting the chromatograms into strips carrying the individual metabolites, and counting the radioactivity associated with the metabolites in a scintillation spectrometer.

RESULTS AND CONCLUSIONS

The results of this experiment are given in Table 12. The data are presented as picomoles of derivative produced during the 30 min incubation period, calculated on the basis of the radioactivity found associated with the known metabolites on the TLC plates. The calculation assumed that the derivatives had essentially the same specific radioactivity as the parent ^{3}H-BaP. The conclusion from the data was that there was no difference between microsomes prepared from frozen liver shipped from the THRU and those prepared from fresh liver at UC Irvine or liver freshly frozen at UC Irvine. Therefore, the method of freezing and shipment of liver to UC Irvine appears to be feasible for future experiments involving rats treated at the THRU with 3-methylcholanthrene. Microsomes were also prepared from kidney and lung tissues; however, the levels of enzyme activity were barely above background, and no definite conclusion can be made regarding the effects of shipment. This latter result is in accord with previous experience, in which little BaP metabolism has been found in these tissues unless the rats had been previously treated with an inducer of the enzyme activity. Examples of such inducers would be BaP itself, 3-methylcholanthrene, and phenobarbital. It is expected that lung and kidney tissues from rats treated with 3-methylcholanthrene at the THRU in future experiments will show significant enzyme activity toward BaP.

The major metabolites identified were 3 dihydrodihydroxy (diol) derivatives and at least 2 monohydroxy derivatives of BaP. The latter included 3-hydroxy-BaP (3-OH-BaP) and 9-OH-BaP, but it was not possible to separate these derivatives on TLC. (Figure 1 shows the numbering scheme for BaP.) In addition to the identified derivatives, some radioactivity remained in the aqueous phase of the incubation mixtures and some at the origin of the TLC plates. Based on previous experience with this system, these unidentified metabolites represent conjugated derivatives of BaP and multiple hydroxylated derivatives, respectively.

FUTURE PLANS

The range-finding studies to establish the correct dosage for tumor induction in the rats are expected to be completed in November or December, 1981. At that time final decisions will be made regarding the numbers of animals and the frequency of sampling for the subsequent tumor study. In the time before December, activity in this laboratory will be directed toward determining the immediate effects of intratracheal MCA on ^{3}H-BaP metabolism by lung and liver microsomes, and whether the intratracheal treatment causes chromosomal damage (i.e., induces sister chromatid exchanges) in lung

TABLE 12

METABOLISM OF ^{3}H-BaP TO HYDROXYLATED DERIVATIVES BY MICROSOMES FROM FRESH AND FROZEN LUNG, LIVER AND KIDNEY OF FISCHER 344 MALE RATS

Metabolism of ^{3}H-BaP was carried out and metabolites separated as described in the text. The values are the average of duplicate samples, and are expressed as picomoles of metabolites produced in 30 minutes.

TISSUE	9,10-DIOL	7,8-DIOL	4,5-DIOL	3-and 9-OH-BaP and QUINONES	UNIDENTIFIED ON TLC PLATE	UNEXTRACTED WATER SOLUBLE	^{3}H-BaP NOT METABOLIZED	PMOLES RECOVERED
FROZEN AT THRU:								
LIVER #1	223	355	250	1417	371	464	4859	7939
LIVER #2	268	313	271	1442	441	486	4871	8092
LUNG	18	17	25	162	39	187	6431	6879
KIDNEY	5	7	8	140	23	98	6531	6812
FROZEN AT UCI:								
LIVER #1	293	381	371	1403	561	683	4215	7907
LIVER #2	228	346	296	1476	460	594	4873	8273
LUNG	14	12	20	137	30	73	6511	6797
KIDNEY	7	7	8	140	22	69	6633	6886
FRESH AT UCI:								
LIVER #1	298	408	365	1332	546	563	4232	7744
LIVER #2	319	240	477	1120	699	560	4600	8015
LUNG	26	22	31	176	29	70	6304	6658
KIDNEY	7	9	9	126	25	116	6614	6906
a BLANK FOR LUNG & KID.	4	5	7	110	19	23	6908	7076
a BLANK FOR LIVER	16	13	23	142	48	15	7919	8176

a The blanks contained all components except the microsomes. The blanks for liver and lung and kidney are different because the ^{3}H-BaP stock solutions are made up separately, and slightly less was used in the incubation mixtures with lung and kidney microsomes. The blank values have not been subtracted from the values obtained with microsomes.

cells. Methods have been established for preparation of primary cell cultures from lungs of treated animals, and these cells will be studied for evidence for chromosomal damage. Experiments are now in progress at UC Irvine in which rats are given MCA intratracheally as a suspension in a manner similar to that used for the induction of lung tumors. The results of studies of ^{3}H-BaP metabolism by the lung microsomes and the frequency of sister chromatid exchanges in lung cells will appear in future reports.

Methods and equipment are now being developed at the THRU to provide for the in vivo labeling of treated rats with ^{3}H-dThd. Lungs of these animals will be fixed by inflation of the lung with buffered 10% formalin, and both paraffin and plastic sections prepared for autoradiographic analysis. These specimens will permit detection of cellular changes in number and in proliferation, and will provide for ultrastructural examination, if considered necessary.

REFERENCES

Brandt, W.N., W.G. Flamm, and N.J. Bernheim, (1972), The Value of Hydroxyurea in Assessing Repair Synthesis of DNA in HeLa Cells, Chem. Biol. Interactions 5:327-339.

Cleaver, J.E., (1974), Repair Processes for Photochemical Damage in Mammalian Cells, In: Advances in Radiation Biology, Volume 4, J.T. Lett, H. Adler, and M.R. Zelle, editors, Academic Press, New York.

Guirgis, H.A., H.T. Lynch, T. Mate, et al., (1978), Arylhydrocarbon Hydroxylase Activity in Lymphocytes from Lung Cancer Patients and Normal Controls, Oncology, 3:105-109.

Hebel, R. and M.W. Stromberg, (1976), Anatomy of the Laboratory Rat, The Williams and Wilkins, Company, Baltimore.

Kaighn, M.E., (1973), Human Liver Cells, In: Tissue Culture. Methods and Applications, P.F. Kruse, Jr. and M.K. Patterson, Jr., editors, pp. 54-58, Academic Press, New York.

Kouri, R.E., T. Rude, P.E. Thomas, and C.E. Whitmire, (1976), Studies on Pulmonary Hydrocarbon Hydroxylase Activity in Inbred Strains of Mice, Chem. Biol. Interactions 13:317-331.

Laishes, B.A., D.J. Koropatnick, and H.F. Stich, (1975), Organ Specific DNA Damage Induced in Mice by the Organotropic Carcinogens 4-Nitroquinoline-1-oxide and Dimethylnitrosamine, Proceedings of the Society of Experimental Biological Medicine 149:978-982.

Painter, R.B. and J.E. Cleaver, (1969), Repair Replication, Unscheduled Synthesis and the Repair of Mammalian DNA, Radiation Research 37:451.

Phillips, H.J., (1973), Dye Exclusion Tests for Cell Viability, In: Tissue Culture. Methods and Applications, P.F. Kruse, Jr. and M.K. Patterson, Jr. editors, pp. 406-408, Academic Press, New York.

Rasmussen, R.E. and I.Y. Wang, (1974), Specific Metabolism of Benzo(a)pyrene Depends upon the Inducer of Hydroxylase Activity, Cancer Research 34:2290-2295.

Rasmussen, R.E., C.H. Boyd, D.R. Dansie, R.E. Kouri, and C.J. Henry, (1981), DNA Replication and Unscheduled DNA Synthesis in Lungs of Mice Exposed to Cigarette Smoke, Cancer Research 41:2583-2588.

Saffiotti, U., F. Cefis, and L.H. Kolb, (1968), A Method for the Experimental Induction of Bronchogenic Carcinoma, Cancer Research 28:104-124.

Scott, J.F., A.P. Fraccastoro, and E.P. Taft, (1956), Studies in Histochemistry: I. Determination of Nucleic Acids in Microgram Amounts of Tissue, J. Histochem. Cytochem. 4:1-10.

Stich, H.F. and D. Kieser, (1974), Use of DNA Repair Synthesis in Detecting Organotropic Actions of Chemical Carcinogens, Proceedings of the Society of Experimental Biological Medicine 145:1339-1342.

Wang, I.Y., R.E. Rasmussen, and T.T. Crocker, (1974), Metabolism of Benzo(a)pyrene by Microsomes from Tissues of Pregnant and Fetal Hamsters, Life Sciences 15:1291-1300.

☆U.S.Government Printing Office: 1981 — 559-007/6003